Journal of Surgical Oncology 63:9–16 (1996)

DNA Ploidy and S-Phase Fraction of Neoplastic and Non-neoplastic Lesions of the Human Gallbladder

TORU YOSHIDA, MD, SHIN-ICHI NAKAMURA, MD, AND TAMOTSU SUGAI, MD
From the Division of Pathology, Central Clinical Laboratory, School of Medicine, Iwate Medical University, Morioka, Japan

Studies on the cell kinetics of the human gallbladder are difficult because of epithelial degeneration by bile. Using the epithelial isolation technique, however, we were able to determine the degree of degeneration and to examine the cell kinetics of gallbladder lesions in freshly resected surgical specimens. Normal and neoplastic epithelia were isolated nonenzymatically from freshly resected gallbladder. The nuclear DNA content and S-phase fraction were estimated in 110 patients with gallbladder lesions by flow cytometry (FCM). Normal tissues and all lesions except carcinomas were diploid. The S-phase fraction of gallstone cases was significantly higher (1.47 ± 0.70%; mean ±SD) than normal (0.79 ± 0.39%) ($P < 0.0006$). All gallbladder carcinomas were multiploid, and their S-phase fraction was 11.63 ± 3.65%. Cell renewal of normal gallbladder is low. In the gallstone cases, the S-phase fraction was increased, possibly correlated with carcinogenesis.

KEY WORDS: **gallbladder carcinoma, epithelial isolation technique, flow cytometry, DNA ploidy, S-phase fraction**

INTRODUCTION

Epithelial cell renewal in the gastrointestinal tract has been studied extensively [1–3], but there are only a few reports on cell kinetics of the human gallbladder [4–7], and little is known about the ways the gallbladder mucosa responds to injury [8]. Studies on cell kinetics in the gallbladder have several obvious difficulties. First, autolysis of the mucosal epithelium occurs within a few hours after death or operation, so cell kinetics is difficult to examine by explant culture, autoradiography, or in vivo bromodeoxyuridine (BrdU) labeling. Second, it is difficult to examine fresh normal human gallbladder as a control.

The DNA content of large numbers of cells can be analyzed rapidly by flow cytometry (FCM). FCM analysis provides other useful information on tumor cells, such their DNA ploidy and the fractions in different phases of the cell cycle. The fractions of cycling cells, especially the S-phase fraction, represent the proliferative activity [9,10], but accurate data cannot be obtained by conventional methods because of contamination of stromal cell nuclei.

The aim of the present study was to examine the biological characteristics of gallbladder lesions. The mucosal epithelial isolation technique enabled us to obtain pure mucosal epithelium uncontaminated by stromal cells [11,12], for measurement of the precise DNA content of isolated mucosal epithelium by FCM. In this way we studied the DNA ploidy and S-phase fraction of neoplastic and non-neoplastic human gallbladder lesions.

MATERIALS AND METHODS

Clinical Materials

Between 1993 and 1994, tissues were taken from 110 patients with gallbladder diseases undergoing resection

Abbreviations: BrdU, bromodeoxyuridine; CMFH, calcium- and magnesium-free Hanks' balanced salt solution; CV, coefficient of variation; EDTA, ethylene-diaminetetraacetic acid; FCM, flow cytometry; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; PI, propidium iodide.

Accepted for publication May 6, 1996.

Address reprint requests to Dr. Toru Yoshida, Division of Pathology, Central Clinical Laboratory, School of Medicine, Iwate Medical University 19-1 Uchimaru, Morioka, 020, Japan.

TABLE I. Clinical Data on Gallbladder Lesions

1. No. of patients: 110	
2. Sex: male-to-female ratio = 61 : 49	
3. Age: mean 60.9 yr (range, 21–85 yr)	
4. Cases of gallbladder lesions	
Normal	16
Normal with OHBPC[a]	22
Stone	36
Polyp	13
Cancer	18
APBDS[b]	3
Others	2

[a]Other hepatobiliary–pancreatic carcinomas.
[b]Anomalous arrangement of pancreaticobiliary ductal system.

of the gallbladder at Iwate Medical University Hospital and related hospitals. The patients consisted of 61 males and 49 females with a median age of 61.2 years (range, 21–85 years). Thirty-six patients had gallbladder stones, 13 had gallbladder polyps, 18 had adenocarcinoma, 22 had a normal gallbladder with other hepatobiliary–pancreatic carcinoma, 3 had an anomalous arrangement of the pancreaticobiliary ductal system, one had an adenomyomatosis, and one patient had a diffuse epithelial hyperplasia of the gallbladder.

Sixteen human gallbladders were used as controls; 15 were resected from patients for prophylactic cholecystectomy during gastrectomy for gastric cancer, and one was resected during operation on a liver abscess. All these gallbladders were macroscopically normal and contained no stones. They showed no inflammation or cholesterosis on microscopic examination. Clinical data are presented in Table I.

Isolation of mucosal epithelia. The method of Nakamura et al. [11,12] was used for isolation of the mucosal epithelium. Briefly, fresh normal epithelium were routinely sampled from three parts, the neck, body and fundus, of the gallbladder. Normal mucosa were separated from muscularis with scissors. Samples of more than 1 cm^3 were obtained from gallbladder carcinoma. They were cut into pieces of 3 mm^3 with a razor and incubated at 37°C for 30 min in calcium- and magnesium-free Hanks' balanced salt solution (CMFH) containing 30 mmol/L ethylene-diaminetetraacetic acid (EDTA). The tissue was then stirred gently in CMFH. Mucosal epithelium was separated from the lamina propria mucosae or fibrous stroma in 30–40 min. The isolated mucosal epithelium was promptly fixed in 70% ethanol and stored at 4°C.

Identification of mucosal epithelium. Samples of fixed mucosal epithelium were observed under a dissecting microscope (FI-150, Olympus, Tokyo). Normal epithelia had a characteristic sheet-like appearance with folds forming valleys and crests; neoplastic epithelia constituted a rather thick cluster. The histology of mucosal epithelium in paraffin-embedded sections stained with H&E confirmed their origin. Normal and neoplastic epithelia were carefully separated and examined independently.

Flow cytometry. The normal and neoplastic epithelia of each patient were incubated with 0.02% pepsin (pH 2.0, Sigma Chemical Co., St. Louis, MO) for 5 min at 37°C, washed twice with 0.2 mol/L phosphate-buffered saline (PBS), and flushed up and down several times in a disposable syringe (1 ml, Nipro, Tokyo) with a 27-gauge needle. Both normal and neoplastic nuclei were stained with propidium iodide (PI) (50 μ/ml) (Sigma) containing ribonuclease (0.25 mg/ml) (Sigma) in 0.2 mol/L PBS. The preparation was filtered through 37-μm nylon mesh (Tokyo Screen, Tokyo), and stained nuclei were analyzed on an Epics Profile flow cytometer (Coulter, Hialeah, FL). An argon-ion laser was used at 15 mW at a wavelength of 488 nm for excitation. Emission was measured using a 620-nm bandpass filter. In each sample, 1×10^4 nuclei were counted.

Analyses of histograms. DNA histograms were analyzed using the software Multicycle (Coulter). We used normal epithelial cells of the same organ and the same individuals as a DNA diploid standard. Samples with at least one separate G0/G1 peak besides the diploid peak were defined as those showing DNA aneuploidy. Samples that had more than one aneuploid stemline with one diploid peak were defined as DNA multiploid. To identify the diploid peak in cases of DNA multiploidy, we prepared a mixture of the normal and the neoplastic nuclei of the patient. After staining with PI, the mixture was analyzed with FCM; the mixture of normal and neoplastic nuclei made one of the peaks higher or formed another distinct peak; that peak was then identified as diploid stemline. From the FCM–DNA histograms, we estimated the proliferative activity by S-phase fraction. The S-phase fraction of all neoplastic and non-neoplastic lesions was calculated by Multicycle, which follows the nonlinear least-squares fitting described by Marquardt and permits subtraction of debris and aggregates [13]. In cases with multiploidy, the average S-phase fractions of diploid and aneuploid populations were calculated and used as the mean S-phase fraction. All samples in this study were examined at least three times, and the samples with the lowest coefficient of variation (CV) were used for DNA analysis.

Histopathological Examination

Sections of all resected surgical materials were routinely embedded in paraffin and stained with H&E for histopathological diagnosis. The tissue fragments remaining after isolation of mucosal epithelia were fixed with 70% ethanol and examined histologically. These fragments showed some remaining epithelial cells and allowed estimation of the characters of the isolated mucosal lesions.

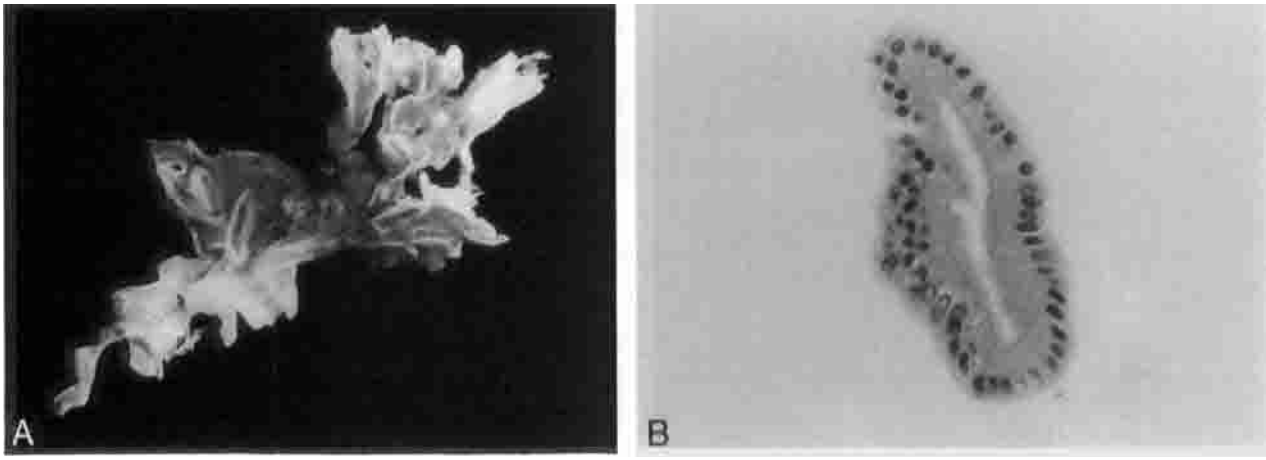

Fig. 1. Mucosal epithelium of normal gallbladder. **A:** Isolated normal epithelium. ×25. **B:** Histologic examination. There is no lamina propria mucosae. H&E stain. ×400.

Statistical Analysis

Statistical analysis was performed with StatView-II software (Abacus Concepts, Berkeley, CA). Statistical differences between mean values in this study were analyzed by the nonparametric Mann–Whitney U-test.

RESULTS

The mucosal epithelium of normal tissue and of carcinoma was easily separated from the lamina propria after incubation with EDTA. In some specimens, such as those of severe cholecystitis, the yield of mucosal epithelium was poor because of mucosal desquamation by inflammation. The three-dimensional structure of the isolated epithelium confirmed that these epithelial cells were not degenerated.

Normal gallbladder had no glandular structures. Mucosal epithelium is arranged in folds that have the appearance of crests and valleys. Under a dissecting microscope, normal mucosal epithelium appeared as a thin sheet with some folds; the fragments examined were up to 3 mm^2 in size (Fig. 1A). No morphological differences was seen under a dissecting microscope in the normal gallbladder epithelia isolated from three separate sites. The epithelial cluster of adenocarcinomas was also sheet-like, but rather thicker (Fig. 2A). The nature of the isolated mucosal epithelium was confirmed in paraffin-embedded histological sections (Figs. 1B, 2B,C). The DNA content of all adenocarcinomas showed multiploidy in FCM histograms. By contrast, all non-neoplastic lesions were diploid.

The overall results of S-phase fraction of gallbladder lesions are summarized in Table II. There were no significant differences of the S-phase fractions in the three parts of normal gallbladder mucosa (Table III). The mean S-phase fraction in gallbladder carcinomas was the highest (11.63 ± 3.65%, range 6.2–18.2%). The mean S-phase fraction was low in normal gallbladder epithelium (0.79 ± 0.39%) and was significantly higher than normal in gallstone cases (1.47 ± 0.63%; $P < 0.0006$). The S-phase fraction values in other cases, such as gallbladder polyps and gallbladder with other hepatobiliary–pancreatic carcinomas, were not significantly different from those in normal gallbladders. In the cases of obstructive jaundice, mucosal hyperplasia of the gallbladder was often observed [14], but the DNA ploidy and S-phase fraction values in these cases were not significantly different from normal. In almost all cases of gallstones, the mucosal epithelium was desquamated and in some cases mucosal folds seemed more numerous. In the gallstone cases, the S-phase fractions of the fundic portion were significantly higher than those in the neck portion ($P < 0.05$), but not significantly different from those in the body portion (Table IV). The mean CV of normal epithelial nuclei was 3.68 ± 0.5% (range, 2.12–4.89%) and that in neoplastic samples was 4.32 ± 1.6% (range, 3.56–6.56%).

Clinicopathological findings and DNA indices with S-phase fractions of gallbladder carcinoma cases are summarized in Table V. Multicycle software makes it possible to calculate the S-phase fractions in multiploidy of each peak separately. The patients, 12 women and 6 men, ranged in age from 38 to 79 years (mean age, 64.5 years). Seven patients had gallstones. All neoplastic samples were multiploid. In cases of gallbladder carcinoma, the diploid peaks could be identified by addition of normal epithelial nuclei of gallbladder of the same individual before PI stainings. Seventeen cases were hyperdiploid, the mean DNA index (DI) being 1.77 (range, 1.36–2.51). One case was hypodiploid with a DI of 0.89. Figure 3 indicates histograms, which could discriminate diploid peak by adding normal nuclei before PI stainings. The

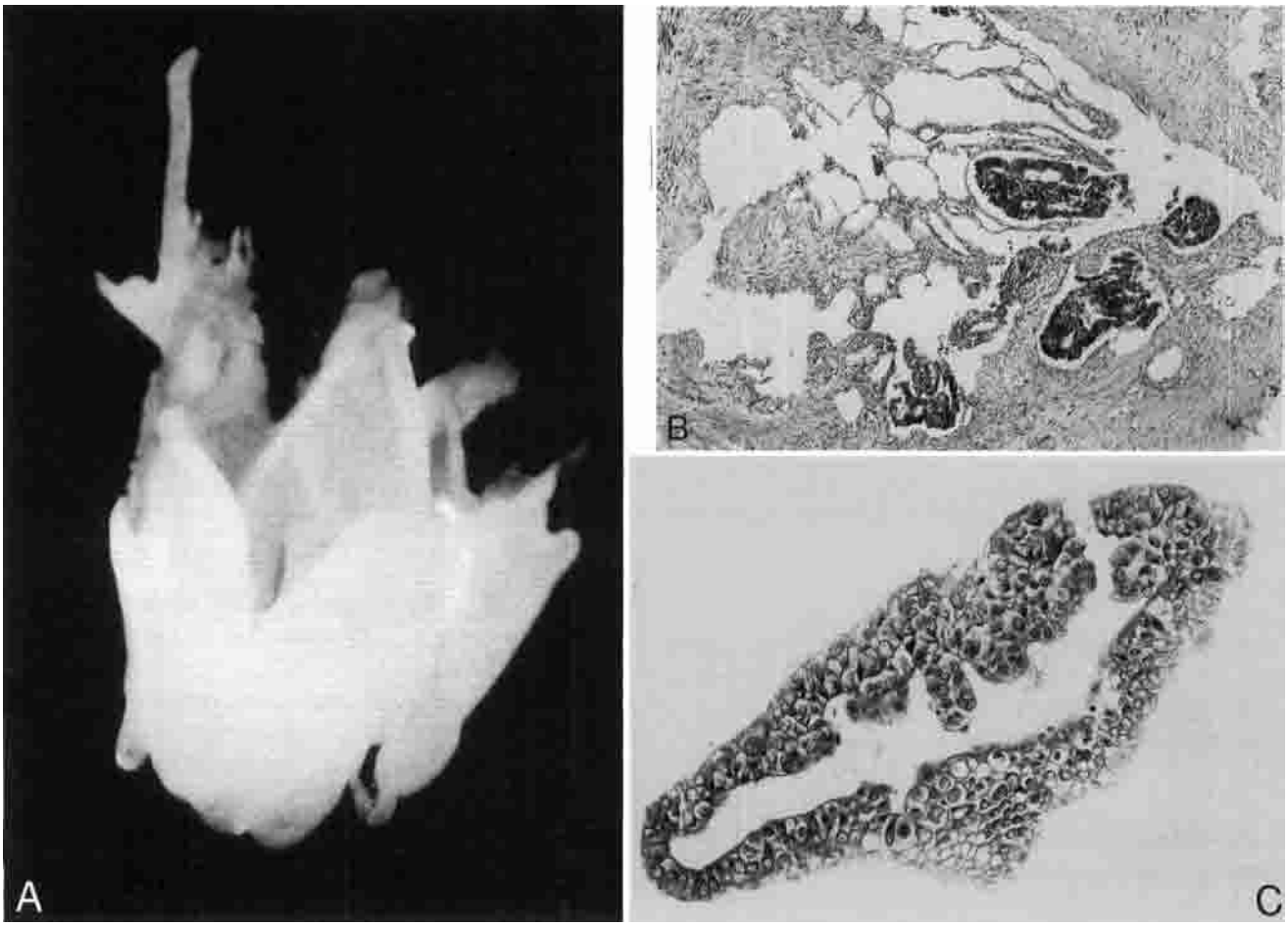

Fig. 2. Epithelial cluster of gallbladder carcinoma. **A:** Epithelial cluster, which is rather thick. ×25. **B:** Photomicrograph of remaining lamina propria of surgical specimens of gallbladder carcinoma after isolation procedure, showing some remaining clusters of carcinomatous epithelium. H&E stain. ×200. **C:** Histologic examination. H&E stain. ×400.

TABLE II. S-Phase Fractions in Gallbladder Lesions (%)†

Normal	0.79 ± 0.39 (0.3–1.7)*,**
Normal with OHBPC[a]	1.13 ± 0.78 (0.4–3.5)
Stone	1.47 ± 0.70 (0.3–3.3)*
Polyp	1.12 ± 0.69 (0.1–2.9)
Carcinoma	11.63 ± 3.65 (6.2–18.2)**
APBDS[b]	1.37 ± 0.58 (0.8–2.0)

†Mean ±SD (range).
[a]Normal gallbladder with other hepatobiliary–pancreatic tract carcinomas.
[b]Anomalous arrangement of pancreaticobiliary ductal system.
*$P < 0.0006$. **$P < 0.0001$ (Mann–Whitney U-test).

value of S-phase fractions of each diploid peak showed high compared with diploid peak of normal samples.

DISCUSSION

Isolated crypts of the human colon have been analyzed by FCM [12,15,16], but no attempts have been made to

TABLE III. S-Phase Fractions in Normal Gallbladder Mucosal Epithelium

1. Sex: male-to-female ratio = 10 : 6		
2. Age: mean 59.3 yr (range, 42–76 yr)		
3. S-phase fraction (%): mean ±SD (range)		
	All	0.79 ± 0.39 (0.3–1.7)
	Neck	0.66 ± 0.34 (0.1–1.2)
	Body	0.77 ± 0.43 (0.2–1.7)
	Fundus	0.81 ± 0.62 (0.2–2.6)

TABLE IV. S-Phase Fractions of Mucosal Epithelium in Cases With Gallstone

1. Sex: male-to-female ratio = 21 : 15		
2. Age: mean 57.1 yr (range, 21–85 yr)		
3. S-phase fraction (%): mean ±SD (range)		
	All	1.49 ± 0.84 (0.3–4.9)
	Neck	1.19 ± 0.64 (0.4–2.6)*
	Body	1.48 ± 0.82 (0.3–3.9)
	Fundus	1.74 ± 1.12 (0.5–4.9)*

*$P < 0.05$ (Mann-Whitney U-test).

TABLE V. S-Phase Fractions of Gallbladder Carcinoma

No.	Age/Sex	Stone	Locus	Histology	pT	pN	pM	Stage	DNA index (S-phase fractions, %)	Mean S-phase fraction (%)
1	62/F	(−)	Gf	PDA	2	Ib	0	III	1.0 (4.1), 1.53 (17.1), 1.79 (11.4)	11.4
2	59/F	(+)	Gfbn	MDA	2	0	0	II	1.0 (27.7), 1.73 (2.1)	15.7
3	41/M	(−)	Gfbn	ASC	4	Ib	0	IV	1.0 (18.6), 1.85 (15.8), 1.97 (17.9)	18.2
4	79/F	(−)	Gb	PAP	2	0	0	II	1.0 (7.4), 1.67 (26.9)	10.9
5	68/F	(−)	Gbf	UN	4	Ia	0	IV	0.89 (4.6), 1.0 (17.8)	13.3
6	76/M	(+)	Gfb	WDA	2	Ib	0	III	1.0 (3.5), 1.56 (18.8), 1.99 (6.7)	6.2
7	63/M	(+)	Gf	PDA	3	0	0	III	1.0 (5.0), 1.64 (13.5)	11.5
8	73/F	(−)	Gf	WDA	2	0	0	II	1.0 (2.5), 1.92 (33.1)	16.3
9	71/M	(−)	C	MUC	3	0	0	III	1.0 (13.1), 1.89 (2.6)	9.1
10	76/F	(+)	Gf	PAP	Ia	0	0	I	1.0 (9.1), 1.98 (20.8)	13.1
11	38/M	(+)	Gfb	WDA	3	Ib	0	III	1.0 (14.0), 1.36 (15.9), 2.51 (11.4)	15.4
12	69/F	(−)	Gbf	PDA	4	0	0	IV	1.0 (6.3), 1.81 (15.5)	9.0
13	53/F	(−)	Gfbn	WDA	Ia	0	0	I	1.0 (5.8), 1.56 (22.3), 1.89 (16.5)	15.0
14	66/F	(−)	Gf	WDA	2	0	0	II	1.0 (27.4), 1.79 (5.8)	7.8
15	71/M	(+)	Gfbn	MDA	4	Ib	0	IV	1.0 (5.0), 1.70 (8.9)	7.0
16	72/F	(−)	Gf	PAP	Ia	0	0	I	1.0 (15.5), 1.97 (3.4)	6.0
17	72/F	(+)	Gfb	WDA	2	0	0	II	1.0 (39.4), 1.52 (13.3)	15.0
18	62/F	(−)	Gf	WDA	Ib	0	0	I	1.0 (13.6), 1.38 (15.9)	14.6

GB, gallbladder; Gb, body of GB; Gf, fundus of GB; C, cystic duct; Gbf, body and fundus of GB; Gfb, fundus and body of GB; Gfbn, fundus, body and neck of GB; WDA, well-differentiated adenocarcinoma; MDA, moderately differentiated adenocarcinoma; UN, undifferentiated carcinoma; MUC, mucinous adenocarcinoma; PAP, papillary adenocarcinoma; PDA, poorly differentiated adenocarcinoma; ASC, adenosquamous carcinoma; pT, pathologic stage of primary tumor; pN, pathologic stage of lymph node metastasis; pM, pathologic stage of distant metastasis.

separate mucosal epithelial cells from the stroma of the gallbladder. One reason is the difficulty of obtaining fresh specimens of gallbladder mucosa without autolysis: the mucosa of human gallbladder is fragile, so it is hard to investigate its cell kinetics. Moreover, by conventional methods, it is difficult to determine the S-phase fraction accurately because of contamination with stromal cells [17,18]. Excessive debris or overlapping S-phase fractions in cases of aneuploidy influenced measurement of S-phase fraction. Furthermore, in diploid tumors, contamination of nonmalignant cells, such as leukocytes, results in an erroneously low S-phase fraction. We believe that the mucosal epithelium isolation technique is an improvement on conventional methods. In this study, we obtained satisfactory results by this technique, using fresh specimens of human gallbladder obtained within 1 hr after operation. By isolation of epithelium, we were able to analyze the DNA content and estimate the S-phase fraction of pure mucosal epithelial nuclei. In addition, using materials fixed in 70% ethanol, repeated DNA analyses of specimens from the same case were possible.

Our data indicated that the epithelial cells of normal human gallbladder are slowly renewed. The mean S-phase fraction of normal epithelium was 0.79%; in some samples it was 0%. Simple tubulo-alveolar glands are located only in the neck portion, and mucous gland metaplasia increases steadily in frequency and severity with age [19]. But these facts were not correlated with morphological differences of the isolated mucosal epithelium seen under a dissecting microscope or by the DNA ploidy patterns or the S-phase fractions of the nuclei. In vivo labeling of human gallbladder by injection of tritiated thymidine has not been undertaken for ethical reasons, but in vitro labeling of surgical specimens with tritiated thymidine makes it possible to observe and count cells showing DNA synthesis. In previous autoradiographic studies in vitro, the labeling of human gallbladder mucosa was 0.14–0.16% [6,7]. However, there are some methodological difficulties in obtaining accurate data by in vitro autoradiography [20].

Putz and Willems [6] suggested from in vitro labeling of mucosal specimens with ^{3}H-thymidine and autoradiography that physical distension stimulated growth of human gallbladder mucosa. Scott [21] reported high DNA synthesis indices in lincomycin-induced experimental gallstone in the guinea pig. Cell renewal of the gallbladder is purported to occur in response to the external stimulation of mucosal desquamation, but details of this response are still unknown.

Cholecystolithiasis has been claimed to increase the incidence of carcinoma. Parkash et al. and many others reported that gallstones are closely correlated with gallbladder carcinoma [22–25], but almost all these reports were based on epidemiological data, and the precise relationship between gallstones and gallbladder carcinoma remains unknown [7]. In the present study, gallstone patients had higher S-phase fraction values than were found in normal gallbladders. This result supports the idea of acceleration of the epithelial cell cycle in gallstone cases and its correlation with carcinogenesis. Inflammation and chronic mechanical irritation of the mucosa by gallstones are probably the main mechanisms responsible for ob-

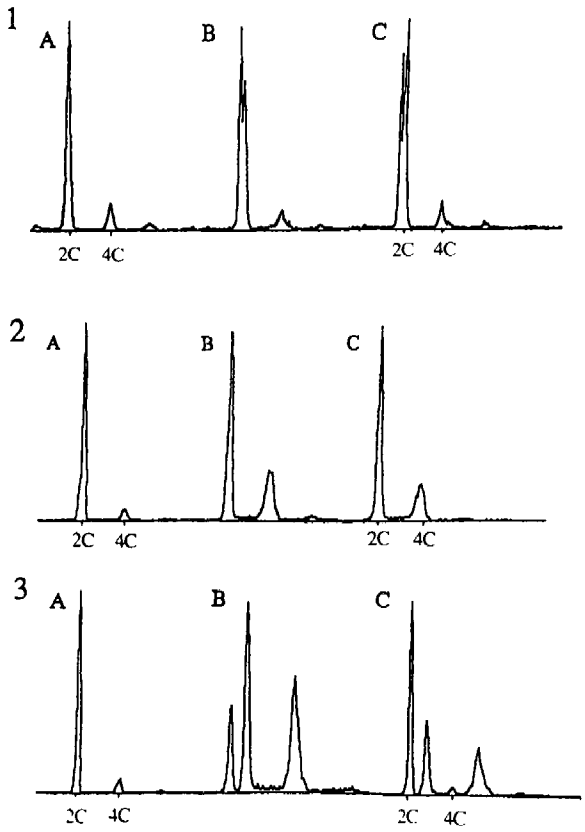

Fig. 3. Histogram. **A:** Normal. **B:** Cancer. **C:** Normal : cancer = 1:1. Histogram 1 (case No. 5): Neoplastic nuclei showed two distinct G0/G1 peaks (B). Right higher peak was considered as a diploid stemline (C), and DNA indices of cancer were 0.89 and 1.0 (normal: CV = 3.03). Histogram 2 (case No. 10): Cancer had two separate peaks. Mixture of normal and neoplastic nuclei made no other peak. DNA indices of cancer were 1.0 and 1.98 (normal: CV = 2.98). Histogram 3 (case No. 11): Cancer had three separate peaks. Left higher peak was considered a diploid stemline, and DNA indices were 1.0, 1.36, 2.51 (normal: CV = 2.98).

served changes in kinetics. But the increased proliferative activity in gallstone cases would be stimulated not only by gallstones but also by intermittent distension, chronic inflammation, and changes of bile salt composition due to diet, drugs, or other causes before and after stone formation [26–28].

The S-phase fraction values in polyp cases (all cholesterol polyps) was not significantly different from normal ones, suggesting a non-neoplastic character of the lining epithelium of cholesterol polyps.

A previous study showed that early gallbladder cancer tended to occur in the fundic portion of the gallbladder [29]. In cases of gallstones, the S-phase fraction of the fundic portion was the highest among three portions (Table IV). These results suggest that mechanical stimulation by gallstones, changes in bile salt composition, and other factors are related to carcinogenesis, especially in the fundic portion. However, the clinical incidence of gallbladder carcinoma in patients with cholecystolithiasis is not sufficient to justify prophylactic cholecystectomy [30–32]: further studies on carcinogenesis are needed to determine its prophylactic value in gallstone patients.

Many investigators have reported the prognostic significance of analysis of DNA ploidy and the S-phase fraction of carcinomas of the esophagus, stomach, colon, lung, breast, and other organs [33–35]. However, there are few reports about DNA ploidy analysis in gallbladder carcinoma. About one-half of cases of gallbladder carcinoma previously reported were aneuploid, the others being diploid [36–39]. Although only 18 cases were analyzed in the present study, all gallbladder carcinomas were multiploid, including diploid and aneuploid stemlines. In multiploid cases that were nearly diploid, it was necessary to obtain a low CV value to differentiate the aneuploid peak from the diploid peak: if CV values are high, aneuploid peaks that are close to diploid peaks may be mistaken as diploid. The CV values in aneuploid cases obtained using the isolation technique were low compared with those in previous reports [36,38,39]. Suto et al. [40] reported a remarkable increase in the frequency of DNA aneuploidy on examination of multiple samples from fresh tumor specimens. The sample size of Suto's study was 2 mm^3 [40]. The sample size of present study was rather large compared with Suto's study. Although multiple sampling sites were not examined in the present study, the volumes of samples might be sufficient to detect several clones in the cancer tissue. The low CV values, sample sizes, and slight debris in FCM histograms in this study are the main reasons for the high frequency of detection of multiploidy.

Yamamoto et al. [36] reported that the ploidy pattern in gallbladder carcinoma was not correlated with either the histological type or tumor stage. The correlation of DNA ploidy and clinicopathological factors could not have been investigated in the present study, as all cases of carcinoma were multiploid. But an interesting finding was that early gallbladder carcinoma showed also multiploid. Thus, change in the DNA content must be an early event in progression of gallbladder carcinoma. Nakamura et al. [12] reported that colorectal carcinomas with a hypodiploid stemline are significantly associated with Dukes' stage C. In this study, undifferentiated adenocarcinoma (small cell type) appeared hypodiploid, including diploid stemlines. The relationship between the prognosis of a patient and DNA ploidy, especially hypodiploidy, requires further study.

A close etiologic association was suggested between anomalous arrangement of pancreaticobiliary ductal system and gallbladder carcinoma [41]. The entry of bile containing pancreatic juice because of regurgitation into the gallbladder is supposed to be the main cause of carcinogenesis. Our three cases with this abnormality showed a slight increase of the S-phase fraction in the gallbladder epithelium. More cases of this anomaly should be ana-

lyzed to clarify the correlation between cell kinetics and carcinogenesis.

The S-phase fraction is a useful indicator of proliferative activity. However, for accurate assessment of tumor growth, we must consider not only DNA synthesis, but also cell loss, because tumor growth is determined by the balance between tumor cell proliferation and cell loss. Steel [42] reported that in human carcinomas cell loss was more than 90%. Unfortunately, the rate of cell loss is still unknown. Further studies are needed to clarify the cell kinetics of gallbladder lesions.

ACKNOWLEDGMENTS

We thank the following persons for supplying fresh tumor samples: Professor K. Saito, M.D., and M. Terashima, M.D., Y. Sugimura, M.D., First Department of Surgery, and the staff of the Division of Pathology, Central Clinical Laboratory, Iwate Medical University; Dr. S. Kanno, Iwate Prefectural Kuji Hospital, Dr. T. Abe, Iwate Prefectural Fukuoka Hospital; and Dr. Y. Mitomo and Dr. H. Abe, Kitakami Saiseikai Hospital. We also thank H. Nakajima for photography.

REFERENCES

1. Lipkin M: Cell replication in the gastrointestinal tract of man. Gastroenterology 48:616–624, 1965.
2. O'Brien MJ, O'Keane JC, Zauber A, et al.: Precursors of colorectal carcinoma: Biopsy and biologic markers. Cancer (Suppl) 70:1317–1327, 1992.
3. Hall PA, Levison DA, Wright NA (eds): "Assessment of Cell Prolifertion in Clinical Practice." London: Springer-Verlag, 1991.
4. Kaye GI, Maenza RM, Lane N: Cell replication in rabbit gallbladder. Gastroenterology 51:670–680, 1966.
5. Scott AJ: Are there proliferative compartments in the gallbladder? Gastroenterology 67:1231–1237, 1974.
6. Putz P, Willems G: Cell proliferation in the human gallbladder epithelium: Effect of distension. Gut 20:246–248, 1979.
7. Putz P, Willems G: Proliferative changes in the epithelium of the human lithiasic gallbladder. J Natl Cancer Inst 60:283–287, 1978.
8. McMinn RMH, Johnson FR: Wound healing in the gallbladder of the cat. Br J Surg 45:76–80, 1957.
9. Hedley DW, Rugg CA, Gelber RD: Association of DNA index and S-phase fraction with prognosis of nodes positive early breast cancer. Cancer Res 47:4729–4735, 1987.
10. Volm M, Hahn EW, Mattern J, et al.: Five-year follow-up study of independent clinical and flow cytometric prognostic factors for the survival of patients with non-small cell lung carcinoma. Cancer Res 48:2923–2928, 1988.
11. Nakamura S, Kino I, Baba S: Nuclear DNA content of isolated crypts of background colonic mucosa from patients with familial adenomatous polyposis and sporadic colorectal cancer. Gut 34: 1240–1244, 1993.
12. Nakamura S, Goto J, Kitayama Y, Kino I: Application of the crypt-isolation technique to flow cytometric analysis of DNA content in colorectal neoplasms. Gastroenterology 106:100–107, 1994.
13. Marquardt DW: An algorithm for least-squares estimation of nonlinear parameters. Soc Ind Appl Math 11:431–441, 1963.
14. Tracy TF Jr., Tector AJ, Goerke ME, et al.: Somatostatin analogue (Octreotide) inhibits bile duct epithelial cell proliferation and fibrosis after extrahepatic biliary obstruction. Am J Pathol 143:1574–1578, 1993.
15. Cheng H, Bjerknes M, Amar J: Methods for the determination of epithelial cell kinetic parameters of human colonic epithelium isolated from surgical and biopsy specimens. Gastroenterology 86:78–85, 1984.
16. Enker WE, Kimmel M, Cibas ES, et al.: DNA/RNA content and proliferative fractions of colorectal carcinomas: A five-year prospective study relating flow cytometry to survival. J Natl Cancer Inst 83:701–707, 1991.
17. Hedley DW, Friedlander ML, Taylor IW, et al.: Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333–1335, 1983.
18. Schutte B, Reynders MMJ, Bosman FT, Blijham GH: Flow cytometric determination of DNA ploidy level in nuclei isolated from paraffin-embedded tissue. Cytometry 6:26–30, 1985.
19. Kozuka S, Hachisuka K: Incidence by age and sex of intestinal metaplasia in the gallbladder. Hum Pathol 15:779–784, 1984.
20. Nakamura S, Kino I, Baba S: Ex vivo autoradiography of the human gastrointestinal tract: A new approach to cell kinetic studies of surgically removed tumour-bearing organs. Jpn J Cancer Res (Gann) 74:116–121, 1983.
21. Scott AJ: Lincomycin-induced cholecystitis and gallstones in guinea pigs. Gastroenterology 71:814–820, 1976.
22. Parkash O: On the relationship of cholelithiasis to carcinoma of the gallbladder and on the sex dependency of the carcinoma of the bile ducts. Digestion 12:129–133, 1975.
23. Lowenfels AB, Lindström CG, Conway MJ, Hastings PR: Gallstones and risk of gallbladder cancer. J Natl Cancer Inst 75:77–80, 1985.
24. Nervi F, Duarte I, Gómez G, et al.: Frequency of gallbladder cancer in Chile, a high-risk area. Int J Cancer 41:657–660, 1988.
25. Albores-Saavedra J, Henson DE: Tumors of the gallbladder and extrahepatic bile ducts. In: "Atlas of Tumor Pathology. 2nd series, fascicle 22. Washington DC: Armed Forces Institute of Pathology, 1986.
26. Takabayashi A, Watkins JB, Soloway RD, et al.: Glycolithocholic acid is greatly increased in stones from patients with carcinoma of the gallbladder. Gastroenterology 79:1058, 1980.
27. Black WC, Key CR, Carmany TB, Herman D: Carcinoma of the gallbladder in a population of Southwestern American Indians. Cancer 39:1267–1279, 1977.
28. Scott AJ: Epithelial cell proliferation in diverse models of experimental cholelithiasis. Gut 19:558–562, 1978.
29. Uchida K, Watanabe H, Yoshida K, et al.: Growth and extension of carcinoma of the gallbladder. Gastroenterol Surg 12:19–30, 1989. (in Japanese)
30. Diehl AK: Gallstone size and the risk of gallbladder cancer. JAMA 250:2323–2326, 1983.
31. Piehler JM, Crichlow RW: Primary carcinoma of the gallbladder. Surg Gynecol Obstet 147:929–942, 1978.
32. Diehl AK: Epidemiology of gallbladder cancer: A synthesis of recent data. J Natl Cancer Inst 65:1209–1214, 1980.
33. Yonemura Y, Ooyama S, Sugiyama K, et al.: Retrospective analysis of the prognostic significance of DNA ploidy patterns and S-phase fraction in gastric carcinoma. Cancer Res 50:509–514, 1990.
34. Sasaki K, Hashimoto T, Kawachino K, Takahashi M: Intratumoral regional differences in DNA ploidy of gastrointestinal carcinomas. Cancer 62:2569–2575, 1988.
35. Sasaki K, Murakami T, Murakami T, Nakamura M: Intratumoral heterogeneity in DNA ploidy of esophageal squamous cell carcinomas. Cancer 68:2403–2406, 1991.
36. Yamamoto M, Oda N, Tahara E: DNA ploidy patterns in gallbladder adenocarcinoma. Jpn J Clin Oncol 20:83–86, 1990.
37. Strom BL, Iliopoulos D, Atkinson B, et al.: Pathophysiology of tumor progression in human gallbladder. Flow cytometry, CEA, and CA19-9 levels in bile and serum in different stages of gallbladder disease. J Natl Cancer Inst 81:1575–1580, 1989.
38. Nishihara K, Tsuneyoshi M: Undifferentiated spindle cell carcinoma of the gallbladder: A clinicopathologic, immunohistochemic, and flow cytometric study of 11 cases. Hum Pathol 24:1298–1305, 1993.
39. Nishihara K, Nagai E, Izumi Y, et al.: Adenosquamous carcinoma of the gallbladder: A clinicopathological, immunohistochemical and flow-cytometric study of twenty cases. Jpn J Cancer Res 85: 389–399, 1994.

40. Suto T, Sasaki K, Sugai T, et al.: Heterogeneity in the nuclear DNA content of cells in carcinomas of the biliary tract and pancreas. Cancer 72:2920–2928, 1993.
41. Kimura K, Ohto M, Saisho H, et al.: Association of gallbladder carcinoma and anomalous pancreaticobiliary ductal union. Gastroenterology 89:1258–1265, 1985.
42. Steel GG: Cell loss from experimental tumors. Cell Tissue Kinet 1:193, 1968.